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Nuclear proteins of hela cells: potential autoantigenic substrate for antinuclear antibodies screening

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KEYWORDS
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Autoimmune disease;
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ABSTRACT

Antinuclear antibodies (ANA) are important in diagnosis and follow-up of patients with autoimmune conditions. The current increase in ANA requests is driven by broadening the use of ANA from a test for lupus to a test for diverse autoimmune diseases, but the standard method is protracted, cumbersome and prone to error. Therefore, simple and reliable testing are needed and autoantigen substrates are required for the development to capture the autoantibodies. This study evaluates the nuclear proteins of Hela cells (NP-HL) as screening marker for systemic autoimmune diseases. Reactivity of 38 ANA positive and 10 negative sera, against NP-HL was determined by western blotting. We demonstrated that NP-HL reacts with 37 ANA positive sera (97%), and without showing any reaction with negative ones. NP-HL was shown to have a diagnostic value as a screening marker for ANA and, therefore, is a suitable alternative substrate for a new antibody test. This research implies that the NP-HL provides a potential to be used as autoantigen substrate in the rapid testing to define ANA for screening of autoimmune diseases.

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Introduction

Autoimmunity is defined as an immune response against self (autologous) antigens and is an important cause of disease. The diseases caused by autoimmunity are called autoimmune diseases[1]. When autoimmune diseases occur, it can cause inflammation, pain, diminished mobility, fatigue and other non-specific symptoms[2]. Autoimmune diseases are chronic diseases in nature and can lead to morbidity and mortality. Therefore, autoimmune diseases are medically and economically important due to high treatment costs, long hospital stays, and early retirement. In the last decade, the incidence and prevalence of autoimmune diseases rise steadily[3]. The estimated prevalence of all autoimmune diseases is about 5-7% of the general population[4].

The presence of autoantibodies in serum against to nuclear antigens such as nucleic acids and intracellular proteins is the hallmark of systemic autoimmune diseases such as systemic lupus erythematosus (SLE), mixed connective tissue disease (MCTD), Sjogren's syndrome (SjS), scleroderma (SS), systemic sclerosis (SSc), and idiopathic inflammatory myopathies (IIMs)[5]. These autoantibodies, also known as antinuclear antibodies (ANA), are predominantly reactive with nuclear antigens. Due to an excellent diagnostic sensitivity of the ANA test, it is considered as the best screening test for these diseases[6,7]. The positivity of ANA screening can make guidance to make confirmatory tests and may be applicable for clarifying an exact clinical diagnosis or prognosis.

Among the currently used ANA screening tests, the indirect immunofluorescence assay (IFA) method applying HEp-2 cells as the substrate is recommended as the gold standard test for ANA detection, with a wide range of advantages and limitations[8-13]. However, there are some challenges such as labor-intensiveness, subjectivity in result interpretation, time-consuming, inadequate reagents standardization and a workforce shortage in clinical laboratories[8-11,14,15]. Therefore, simple and reliable testing are needed for early diagnosis and effective treatment of autoimmune diseases, thereby improving productivity of health care systems by minimizing costs, time, and errors. If available, rapid testing for ANA can improve diagnostics in primary, urgent/emergency, and remote care clinics and enhance medical intervention for patients[16].

For the development of rapid testing, autoantigen substrate is required to capture the autoantibodies. Hela cell is the first continuous cancer cell line. Through application of Hela cells and the various other cell lines, lots of knowledge aspect to human cells have already been perceived. Among thousands of human cancer cell lines, Hela cell is still the most commonly used cell line in biomedical research. It is also non-fastidious nature to grow and has large nucleus which shows a wide range of nuclear antigens correlated with systemic autoimmune diseases and non-expensive. In addition, in concerned of Hep-2 cells substrate, some clinically important autoantibodies can be missed, like SS-A/Ro60 is missed by Hep-2 cells due to the low cellular abundance of this particular protein on Hep-2 cells. Thus, the aim of this study is to evaluate the diagnostic value of nuclear proteins of Hela cells (NP-HL) to be used as screening marker for total ANA.

Materials and methods

Human serum samples

Patient sera were obtained from the patients under auspices of a human subject protocol, approved by the Center for Ethics in Human Research, Khon Kaen University (HE631169). The patients were diagnosed at the Srinagarind Hospital, Khon Kaen, Thailand with ANA positive and titer was determined by indirect immunofluorescence assay (IFA-ANA). All samples were assigned number codes and used in the experiments without knowledge of clinical laboratory information. The IFA-ANA positive sera with clinically significant pattern, homogeneous, speckled, centromere and nucleolar, were chosen and the titer was equal or grater than 1:80. For the negative sera, the samples which showed IFA-ANA negative or the titer was lower than 1:80 were selected.

Cultivation of Hela cells

The Hela cells used in this study were cultured in standard plastic flasks with basic growth medium (Dulbecco's Modified Eagle Medium) supplemented with 10% (v/v) heat-inactivated fetal bovine serum and 1% (v/v) Penicillin-Streptomycin. The cells were maintained in a humidified atmosphere containing 5% CO2 at 37°C.

Nuclear extraction of Hela cells

Nuclear and cytoplasmic protein fractions were collected using the nuclear extraction kit (ab113474; Abcam, UK) In brief, the cells were lysed with cytoplasmic extraction buffer (CEB) and centrifuged at 12,000 rpm for 1 minute to separate the supernatant containing the cytoplasmic proteins and nuclear pellet. Nuclear proteins were extracted from the nuclear pellet with the aid of nuclear extraction buffer (NEB) and centrifugation at 14,000 rpm for 10 minutes. The protein concentration of the nuclear extracts and cytoplasmic extracts were measured. Then, the nuclear extracts and cytoplasmic extracts were used immediately or stored at -80°C until use.

Purity checking of nuclear and cytoplasmic proteins

Nuclear and cytoplasmic proteins extracted from Hela cells were prepared for purity checking by western blotting. In brief, after electrophoresis, gels were electroblotted onto a 0.45-µm pore size polyvinylidene fluoride (PVDF) membrane (10600023, GE Healthcare, UK) which was subsequently blocked in 5% skimmed milk in 0.1% tween 20-TBS (t-TBS) for 1 h at room temperature before incubating with anti-histone H3 antibody (1:1000; ab1791; Abcam, UK) and anti-beta actin antibody (1:5000; ab227387; Abcam, UK). The secondary goat anti-rabbit IgG H & L horseradish peroxidase labeled antibody (ab6721; Abcam, UK) was visualized by the Enhanced Chemiluminescence (ECL) plus reagent (GE Healthcare, UK) using an Amersham imager 600.

Western blotting to detect the reaction of human serum and extracted nuclear proteins from Hela cells (NP-HL)

NP-HL (20, 10, 5, 2 & 1µg) were heated in sample buffer and separated at room temperature by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). After being transferred to PVDF membrane for 1 h at 300 V and 90 mA, the membranes were blocked for 1 h with 5% skimmed milk in t-TBS and sequentially incubated for overnight at 4 °C with 100X diluted IFA-ANA sera, followed by 5,000X diluted of horseradish peroxidase conjugated goat anti-human IgG secondary antibody (Cat-A00166, GenScript, USA) for 1 h at room temperature. Blots were washed 3X for 10 min between steps with t-TBS. Bound antibodies were detected by using Enhanced Chemiluminescence (ECL) plus reagent (GE Healthcare, UK) and quantified on an Amersham imager 600. Anti-histone antibody was used as positive control in the uniform condition to assure the accuracy and quality of the results.

Results

Purity checking of extracted nuclear and cytoplasmic protein

Western blotting results from cellular extracts of Hela cells were shown in figure 1, which indicated that nuclear proteins were sufficiently separated from cytoplasmic proteins. The nuclear marker, histone H3, was highly expressed in nuclear fractions but low amount in cytoplasmic fractions. Conversely, the cytoplasmic marker, ß actin, was detected only acceptable amount in nuclear fraction while it was detected higher concentration in cytosolic portion.

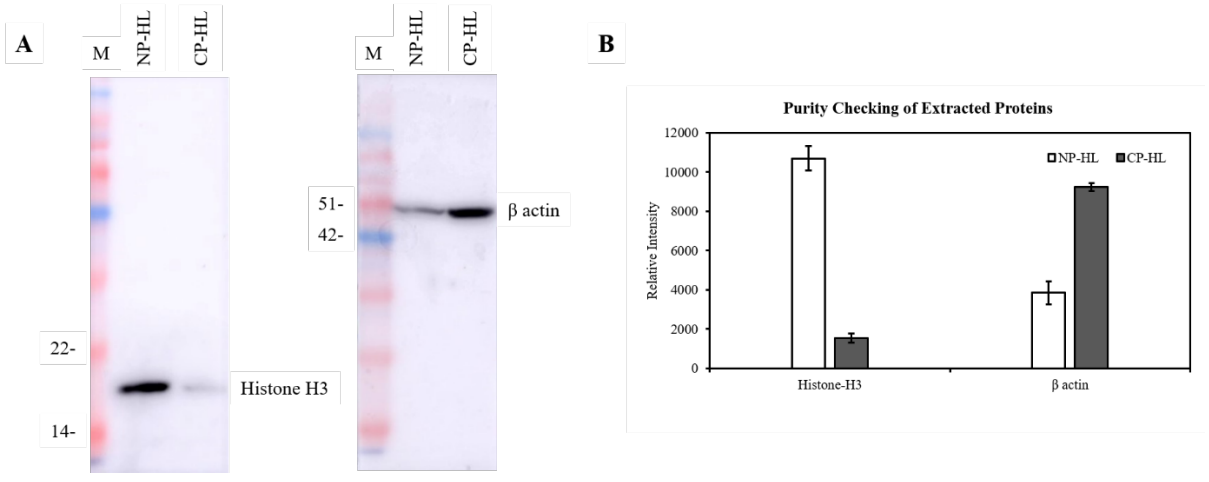

Figure 1 Purity checking of extracted nuclear and cytoplasmic protein from Hela cells (A) Western blotting of extracted nuclear and cytoplasmic protein from Hela cells (NP-HL and CP-HL, respectively) probed with anti-histone H3 antibody (nuclear protein marker) and anti-β actin antibody (cytoplasmic protein marker). (B) From the western blotting result, the in tensity of the western blotting band from NP-HL and CP-HL with anti-histone H3 and anti-β actin was determined. The experiments were performed for three times and data were expressed as mean±SD. (M = molecular weight markers)

Evaluation the reactivity of NP-HL with ANA negative and positive sera

To determine the optimal reactive concentration of NP-HL, western blot analysis was performed using 1, 2, 5, 10, 20 µg of extracted nuclear protein against positive and negative ANA sera which tested with IFA-ANA testing. All proteins of NP-HL in the SDS-PAGE were demonstrated in figure 2A. As could be seen in figure 2B, autoantibody reactivity increased proportionally to the amount of protein bound to the membrane and the result indicated that 20 µg of NP-HL provided the strongest binding ability of autoantigens and antibodies without showing cross-reactivity in negative serum. Therefore, for the rest of the study, 20 µg of NP-HL was routinely used as the optimal reactive concentration of autoantigens.

Evaluation NP-HL with ANA negative and positive samples

NP-HL was tested with the IFA-ANA sera, negative (n = 10) and positive (n = 38) samples, by western blotting. Among the various patterns observed in ANA-positive cases, homogeneous pattern (n = 13), speckled pattern (n = 10), centromere pattern (n = 8) and nucleolar pattern (n = 7) were observed. In western blotting, all negative sera (100%) were observed no detectable band revise and 37 out of 38 positive samples (97%) were detected at least one band. One sample (Homogeneous pattern) was not detected with NP-HL. Additionally, the western blotting results showed the different band in each sample that is shown in table 1. Protein at molecular weight 60 was found to be the most prevalent antibody which is the representation of SS-A/ Ro60 antigen.

Table 1 Serological frequencies of autoantibodies in IFA-ANA patterns

MW in WB (kD)	number (percent)					Autoantibody to*
	Homogeneous (n=13)	Speckled (n=10)	Centromere (n=8)	Nucleolar (n=7)	Total (n=38)	
60	4 (30.8)	1 (10)	2 (25)	3 (42.9)	10 (26)	SS-A/Ro60
52	7 (53.8)	0	2 (25)	0	9 (23.7)	SS-A/Ro52
48	1 (7.7)	3 (30)	2 (25)	2 (28.6)	8 (21)	SS-B/La
33/70	6 (46.1)	1 (10)	0	1 (14.3)	8 (21)	U1-nRNP
60-72	1 (7.7)	1 (10)	5 (62.5)	1 (14.3)	8 (21)	
29	1 (7.69)	4 (40)	1 (12.5)	0	6 (15.8)	Sm
12-24	3 (23.1)	2 (20)	0	0	5 (13.2)	Histone
80	0	0	5 (62.5)	0	5 (13.2)	CENP-B
120	0	1 (10)	3 (37.5)	0	4 (10.5)	CENP-C
135	0	0	1 (12.5)	3 (42.9)	4 (10.5)	RNAP-III
100	3 (23.1)	0	0	0	3 (7.9)	Topoisomerase-I
80	1 (7.7)	2 (20)	0	0	3 (7.9)	Ku
75/100	0	0	0	2 (28.6)	2 (5.3)	PM/Scl
262	2 (15.4)	0	0	0	2 (5.3)	Nucleosome
140-180	0	1 (10)	1 (12.5)	0	2 (5.3)	
37	1 (7.7)	0	0	0	1 (2.6)	Rib-P

*reference number 17

Discussions

Autoantibodies are hallmarks of autoimmune diseases, and testing for total ANA has become a valuable tool at both primary care and subspecialty settings as a trigger for further clinical investigation. Standard ANA testing is performed by indirect immunofluorescence microscopy in centralized clinical laboratories by technicians trained in carrying out its multiple steps and in interpreting fluorescence microscopy images. As a screening tool, the ANA patterns can guide confirmatory testing useful in elucidating a specific clinical diagnosis or prognosis. However, routine use of IFA-ANA testing as a global screening test is hampered by its labor-intensiveness, subjectivity, and limited diagnostic specificity. Thus, the need for simple testing which will be easy to perform and reliable is still a burning issue and the autoantigen substrates with high diagnostic values are required to apply in development of the testing. In this context, we developed and evaluated the extracted nuclear protein from Hela cell with the clinical sample.

The results of western blot analysis clearly established the value of the reactivity of NP-HL for ANA detection, showing strong positive bands on ANA positive sera, versus showing no reaction on all ANA negative sera. In IFA-ANA, some clinically important autoantibodies can be missed, as SS-A/Ro60 was missed by Hep-2 cells due to the low cellular abundance of this particular protein on Hep-2 cells. In contrast, SS-A/Ro60 was highly expressed in NP-HL and was found as the most prevalent autoantibodies in our study. This may be due to the use of extracted autoantigenic nuclear proteins with less content of cytoplasmic proteins. Therefore, autoantigenic targets were highly expressed and masking effect of cytoplasmic proteins to target proteins was also inhibited.

Consequently, NP-HL was evaluated as potential autoantigenic substrate for total ANA detection in western blotting, providing important autoantigenic targets for autoantibodies from cheap source.

In comparison of the results of IFA-ANA and western blotting, high concordance data were found out in negative samples (100%) and positive samples (97%). One sample (homogeneous pattern) was not detected with NP-HL. This difference may be due to the inter-operator variability of performing the different assays and result interpretation. In addition, although our NP-HL contained the major autoantibody targets, including SS-A, SS-B, U1-nRNP, Sm, histone, CENP, RNAP, Topoisomerase-I, Ku, PM/Scl, nucleosome and ribosomal protein, some important autoantigens may not be present. Moreover, this positive sample showed the low titer with IFA-ANA. To understand the association of IFA patterns with specific autoantibodies, our finding was compared to standard reference[17], and found to be very similar with published literature. The speckled pattern showed an association with Sm and SS-B/La which was similar to other studies. Likewise, the centromere pattern is shown in association with the CENP-B & C antibodies and nucleolar pattern with RNAP and PM/Scl antibodies, in accordance with other published studies. However, there are some exceptions, like histones in speckled pattern, SS-A/Ro and U1-nRNP in the homogeneous pattern, and 60-72kD protein in centromere pattern which may be due to the use of different detection techniques. These differences may also be due to the subjectivity and the inter-operator variability of performing the assay and identification the patterns. These technical differences have been observed earlier in literature, and this is one of the major limitations of using IFA as a screening assay for the detection of autoantibodies. Although the validation studies have been performed for automated IFA[18,19], they are not routinely used in developing countries due to the expenses involved. This correlation is very helpful in predicting a specific antibody with a particular ANA pattern and also reminding of limitation in patterns identification of IFA. However, in our study, nuclear antigens were focused and cytoplasmic antigens that binds to cytoplasmic organelles such as mitochondria and the Golgi complex were limited to be identified.

The diagnostic value of the NP-HL is related for screening of total ANA and, warrants further work to set up and validate an autoantibody clinical test for systemic autoimmune diseases based on this autoantigen.

Conclusion

We described the NP-HL as a screening marker for total ANA with potential diagnostic value, strengthened by reactivity evaluation against clinically important ANA sera. This research implies that the NP-HL provides a potential to be used as alternative autoantigen substrate in defining antinuclear antibodies (ANA) for screening of systemic autoimmune diseases.

Take home messages

Our study provides the antigenic avidity of extracted nuclear proteins of Hela cells in capturing antinuclear antibodies. Further study for application of this substrate in method development of ANA screening is warranted.

Conflicts of interest

The authors declare no conflict of interest.

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